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Elevated Cyclic-AMP represses expression of Exchange Protein Activated by cAMP (EPAC1) by inhibiting YAP-TEAD activity and HDAC-mediated histone deacetylation

Short title: Cyclic-AMP represses EPAC1 expression

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ABSTRACT

Ligand-induced activation of Exchange Protein Activated by cAMP-1 (EPAC1) is implicated in numerous physiological and pathological processes, including cardiac fibrosis where changes in EPAC1 expression have been detected. However, little is known about how EPAC1 expression is regulated. Therefore, we investigated regulation of EPAC1 expression by cAMP in cardiac fibroblasts.

Elevation of cAMP using forskolin, cAMP-analogues or adenosine A2B-receptor activation significantly reduced EPAC1 mRNA and protein levels and inhibited formation of F-actin stress fibres. Inhibition of actin polymerisation with cytochalasin-D, latrunculin-B or the ROCK inhibitor, Y-27632, mimicked effects of cAMP on EPAC1 mRNA and protein levels. Elevated cAMP also inhibited activity of an EPAC1 promoter-reporter gene, which contained a consensus binding element for TEAD, which is a target for inhibition by cAMP. Inhibition of TEAD activity using siRNA-silencing of its co-factors YAP and TAZ, expression of dominant-negative TEAD or treatment with YAP-TEAD inhibitors, significantly inhibited EPAC1 expression. However, whereas expression of constitutively-active YAP completely reversed forskolin inhibition of EPAC1-promoter activity it did not rescue EPAC1 mRNA levels. Chromatin-immunoprecipitation detected a significant reduction in histone3-lysine27-acetylation at the EPAC1 proximal promoter in response to forskolin stimulation. HDAC1/3 inhibition partially reversed forskolin inhibition of EPAC1 expression, which was completely rescued by simultaneously expressing constitutively active YAP.

Taken together, these data demonstrate that cAMP downregulates EPAC1 gene expression via disrupting the actin cytoskeleton, which inhibits YAP/TAZ-TEAD activity in concert with HDAC-mediated histone deacetylation at the EPAC1 proximal promoter. This represents a novel negative feedback mechanism controlling EPAC1 levels in response to cAMP elevation.

Keywords: cAMP, EPAC, YAP, TEAD, HDAC, ACTIN

ABBREVIATIONS

α -SMA	Alpha Smooth Muscle Cell Actin
cAMP	3'5'- cyclic adenosine monophosphate
EC	Endothelial cell
EPAC1	Exchange Protein Activated by cAMP
HDAC	Histone Deacetylase
TAZ	Transcriptional Co-Activator With PDZ-Binding Motif
TEAD	TEA Domain Transcription Factor 1
YAP	YES-Associated Protein

INTRODUCTION

Exchange protein activate by cAMP-1 (EPAC1) is a cAMP-dependent guanine nucleotide exchange factor (GEF) that is implicated in numerous physiological and pathological processes [1, 2]. The development of EPAC-selective cAMP analogues such as 8-(4-chlorophenylthio)-2'-*O*-methyl-cAMP[3] has helped identify many important physiological functions of EPAC activity in cardiac cells, including regulation of cardiac fibroblast proliferation[4] and migration[5], regulation of vascular permeability[6-8], regulation of myocyte calcium signalling[9], regulation of myocyte contraction[10], regulation of inflammation[11, 12] and hypertrophy[13] and cardiac fibrosis [14]. Two isoforms of EPAC have been identified, EPAC1 and EPAC2 that respectively contain one or two cyclic AMP binding domains (CBD), which are homologous to those present in cAMP-dependent protein kinase (PKA) regulatory subunits, and allow activation of GEF activity in response to physiological levels of intracellular cAMP [15]. EPAC1 is more widely expressed than EPAC2, being ubiquitously expressed in cardiac cells [5, 16].

Cardiac fibroblasts (CFs) are the main non-myocyte cell type in the heart[17]. They occupy the myocardial interstitium [18], performing a number of important functions that maintain normal myocardial homeostasis, including regulation of myocardial extracellular matrix (ECM) and myocardial integrity as well as functioning as mechano-electric transducers [19]. In response to a range of signals, including angiotensin II and TGF- β , CFs trans-differentiate into myofibroblasts, characterised by increases in alpha-smooth muscle actin (α -SMA) expression, proliferation, migration and collagen synthesis[20]. The transition to this myofibroblast phenotype promotes myocardial healing after cardiac injury [21, 22]. However, aberrant or excessive ECM deposition and myofibroblast proliferation has been implicated in the development of maladaptive fibrosis,

which increases the stiffness of the myocardium, impairs cardiac function and promotes the development of heart failure[20, 23].

Several lines of evidence suggest that signals that elevate intracellular cAMP, such as the extracellular nucleoside adenosine, have been shown to have many anti-fibrotic properties[24] , including inhibition of CF proliferation[25] and collagen synthesis[26]. For example, in a rat model of myocardial infarction, agents that elevate adenosine levels reduced tissue scar formation [27]. These anti-fibrotic properties are mediated via the Gs coupled adenosine A2B receptor, which activates adenylate cyclase (AC) and increases intracellular cAMP levels. Silencing of the adenosine A2B receptor increases CF proliferation[28] and collagen synthesis[29] whereas its overexpression has the opposite effects[28]. These protective effects of adenosine are mediated by increases in intracellular cAMP and activation of EPAC1. For example, over expression of AC or its activation with forskolin inhibits TGF- β - or angiotensin-II induced myofibroblast differentiation, collagen synthesis and proliferation in CF [30-32]. Direct activation of EPAC1, with the selective EPAC agonist, 8-pCPT-2'-O-Me-cAMP or via EPAC1 over expression attenuates angiotensin-II- and TGF- β -induced CF proliferation, migration and collagen synthesis *in vitro*[14, 26, 33] and reduces cardiac fibrosis in a canine model of ventricular tachypacing[14]. Furthermore, pharmacological inhibition of EPAC activity with ESI-09 reverses the inhibitory effects of adenosine on angiotensin-II or endothelin-1 (ET1) induced collagen synthesis and myofibroblast differentiation [20, 34]. Taken together, these data suggest that activation of EPAC1 in response to elevated cAMP inhibits cardiac fibrosis.

Importantly, several studies have demonstrated that EPAC1 levels can be modulated in response to various physiological and pathological stimuli. For example, TGF- β and angiotensin II both promote the down regulation of EPAC1 mRNA and protein expression in cardiac fibroblasts *in vitro* [5, 33]. EPAC1 mRNA and protein levels are also reduced *in vivo* in a rat myocardial infarction model and a canine ventricular tachypacing model of heart failure [14, 33]. Here we characterise the regulation of EPAC1 gene expression in response to cAMP elevation. We describe a novel negative feedback loop by which EPAC1 and PKA activation by cAMP reduces EPAC1 expression by inhibiting TEA Domain Family Transcription Factors (TEAD) activity and histone-H3 lysine-27-acetylation of the EPAC1 proximal promoter.

METHODS

Materials: All chemicals were obtained from Sigma unless otherwise stated. Antibodies to EPAC1 (#4155) were from Cell Signalling Technologies. Antibodies to GAPDH (#MAB374) were from Merck-Millipore. Forskolin and dibutyryl-cAMP was purchased from Sigma Aldrich. BAY60-6583 was purchased from Cambridge Bioscience (U.K.). 6-BNZ-cAMP-AM [35], a cell permeable selective PKA agonist, and 8-pCPT-2'-O-Me-cAMP-AM (8-CPT-AM), a cell permeable selective EPAC super-agonist [36], were purchased from Biolog (Germany) and used at 20 μ M in accordance with the manufacturers recommendations.

Cardiac fibroblast culture: All experiments were performed using different batches of cells that were prepared from different animals. Male Sprague Dawley rat pups were killed by cervical dislocation in accordance with Directive 2010/63/EU of the European Parliament, under schedule 1 of the United Kingdom Home Office Animal Scientific Procedures Act 1986 and performed in accordance with guidelines, regulations and approval of the University of Bristol. Approval was granted by the University of Bristol ethical review board. Cultures of rat cardiac fibroblasts were prepared as previously described [37]. Briefly, hearts from 2- to 3-day-old rats were digested with four cycles of incubation in 0.1% trypsin containing 0.02% EDTA in PBS. Digestion was stopped by the addition of foetal calf serum (FCS) to 20%. The dispersed cells were resuspended in MEM supplemented with 10% FCS, 100 μ g/mL streptomycin, and 100 U/mL penicillin and plated for 1 hour to allow fibroblasts to adhere. The unattached myocytes were removed with two washes in PBS. Cardiac fibroblasts were cultured in Advanced DMEM/F12 supplemented with 100 μ g/ml streptomycin and 100 U/mL penicillin, 10% FCS and 2 mM L-glutamine. H9C2 cardiomyocyte cells were cultured in DMEM supplemented with 100 μ g/ml streptomycin and 100 U/mL penicillin, 10% FCS and 2 mM L-glutamine. Stimulations were performed in serum free DMEM unless otherwise stated. Cells were serum-starved for 18 hours or 4 hours, as indicated in figure legends.

Plasmids and adenoviral vectors: EPAC1 promoter fragments were generated by PCR using KOD polymerase from human genomic DNA. The location of the 800 bp promoter fragment is chr12:48152755-48153554 (Hg19). The proximal TEAD element (5'-GCATTCCTC-3') was mutated to (5'-GCgTgtCTC-3') by PCR with a reverse primer incorporating the mutated element. Promoter fragments were cloned into the Nhe1 sites of the secreted nano-luciferase reporter gene plasmid pNL3.3[*secNluc*/minP] (Promega). The synthetic TEAD luciferase reporter plasmid containing eight copies of a TEAD binding element has been described previously [38] and was

obtained from Addgene (plasmid #34615). Luciferase reporter plasmid containing the minimal TNT promoter was generated by BglII and KpnI digestion of the TEAD-LUC vector to remove the eight TEAD elements, leaving just the minimal TNT-promoter. Plasmid expressing dominant-negative TEAD in which the YAP-binding domain of TEAD is replaced with the repressor domain of Engrailed (TEAD-ENRD) was a gift from Prof Domenico Flagiello (University of Paris Diderot) and has been described previously [39]. Adenovirus vectors expressing constitutively active YAP (YAP_{S127A}), constitutively active TAZ (TAZ_{5SA}) or control virus lacking a transgene (Ad:Control) have been described previously [40]. Cardiac fibroblasts were infected with 1×10^7 pfu/mL of recombinant adenovirus for 18 hours.

Quantitative RT-PCR and Western blotting: Quantification of mRNA and protein levels was performed by RT-qPCR and western blotting respectively, as described previously [41]. Total RNA was extracted using Ambion Pure-Link kits (Thermo Fisher) and was reverse transcribed using the QuantiTect RT kit (Qiagen) and random primers. Quantitative PCR was performed using Roche SYBR Green and a Qiagen Roto-Gene qPCR machine (20'@95°C;20'@62°C;20'@72°C). Primer sequences are described in Supplement Table 1. Western blots were performed using a Mini-Protean II system. Proteins were transferred to PVDF membrane using wet transfer and detected using ECL (Luminata Forte, Millipore) and a digital ChemiDoc imaging system (Bio-Rad).

Transient transfection and reporter gene assays: EPAC1 promoter activity was determined by quantifying the secreted nano-luciferase reporter activity in cells transfected with EPAC1-NLUC. Plasmid transfection was performed by electroporation of 1×10^6 cardiac fibroblasts with 5 µg of plasmid DNA using an Amaxa Nucleofector-1.5 (program A-024). For gene silencing, cells were transfected with 100 pmoles of Silencer Select siRNA (Life Technologies) targeting YAP1 (ID:s170198 and ID:s170200) or TAZ/WWTR1 (ID: s148961) using the standard Nucleofector program A-024. Cells were stimulated with the indicated agents 24 hours post transfection. The next day cell lysates were assayed for firefly luciferase activity and cell culture media assayed for secreted nano-luciferase. Firefly luciferase was quantified using the luciferase assay system (Promega) and secreted nanoluciferase activity assayed using the NanoGlo assay system (Promega) according to the manufactures instructions using Glomax Discover luminometer (Promega).

Electromobility shift assays: Electromobility shift assays (EMSA) were performed using the Lightshift EMSA kit (Thermo Fisher), according to the manufacturer's instructions. Nuclear extracts from 5×10^6 control or pRK-myc-TEAD1 (Addgene #33109) transfected cells were

prepared using the NE-PER nuclear extraction kit (Thermo Fisher). Double stranded 5' biotinylated probes corresponding to the wild-type or TEAD element mutant human EPAC1 promoter TEAD element (Wild-type sense 5'-Biotin- TTC CCC TAC GCA TTC CTC TAC CGT AA-3'; Wild-type antisense 5'-Biotin-TTA CGG TAG AGG AAT GCG TAG GGG AA-3'; TEAD-mutant sense 5'-Biotin- TTC CCC TAC GCg Tgt CTC TAC CGT AA-5' and TEAD mutant antisense 5'-Biotin-TTA CGG TAG Aga cAc GCG TAG GGG AA-3') were synthesised by Sigma Aldrich. 20 fmol of double stranded DNA oligo was used in 20 uL binding reactions according to the Lightshift assay kit protocol. DNA complexes were resolved on 5% non-denaturing polyacrylamide/0.5xTBE gels at 100V. Oligonucleotides were transferred to Hybond-N nylon membrane (Amersham) in 0.5 x TBE at 400 mA for 1 hour at 4°C. Oligonucleotides were detected using the Lightshift kit Extravidin-HRP detection system.

Chromatin Immunoprecipitation assays: Cardiac fibroblasts (approximately 8×10^6 cells) were washed in PBS and fixed in 11% formaldehyde in PBS for eight minutes at room temperature. Formaldehyde was quenched by addition of glycine to a final concentration of 250 mM. Chromatin was prepared using the iDeal ChIP-qPCRkit (Diagenode) according to the manufacturer's instructions. Chromatin was sheared using 2.5 cycles (10 rounds of 30 seconds on and 30 seconds off per cycles) of sonication in a Bioruptor sonicator (Diagenode). Sheared chromatin was immunoprecipitated overnight at 4°C with either 1 µg of anti-H3K27Ac antibody (#C15410174; Diagenode) or an equal amount of rabbit non-immune IgG (#C15410206; Diagenode) according to the iDeal Chip-qPCR kit instructions. Following washing and elution, immunoprecipitated DNA was quantified using qPCR with primers (Forward 5'- CCT CTG GAC TTG GAC TGG TCA TGC-3' and Reverse 5'- GCC GAA TTC CAG CCA GAA CTG AGA-3') amplifying the proximal EPAC located at chr7:139254198-139254358 (rn6)

RAP1^{GTP} activity assays: Levels of cellular RAP1^{GTP} were quantified using the Cell Signalling Active Rap1 detection kit according to the manufacturer's instructions. Briefly, cells were seeded at 5×10^6 cells/well in 6 well plates. Cells were pre-treated with 25 µM forskolin for 24 hours, as indicated before an acute stimulation the following day. RAP1^{GTP} present in cell lysates was bound to 20 µg of GST-RAL-GDS protein and affinity isolated using glutathione resin. Following washing to remove non-specifically bound protein, specifically bound RAP1^{GTP} as eluted in Laemmli sample buffer and analysed for RAP1 protein levels by Western blotting.

F-Actin:G-Actin ratio assay: F:G actin ratio was quantified essentially as previously described [42]. Following treatment, G-actin was extracted in G-actin extraction buffer (PBS, 10%

glycerol, 0.1% triton X-100, 1 mM ATP and complete protease inhibitor) and incubated for 5 min at room temperature with slight agitation. Samples were centrifuged at 15,000 g at 4 °C for 5 min. The supernatant (soluble G-actin) was collected. Triton-X-100 insoluble material (F-actin) remaining in the wells and pelleted from the soluble fraction was lysed in reducing Laemmli SDS sample buffer. Samples were analysed by western blotting using a β -actin specific antibody (Sigma).

Image Analysis: Immunofluorescent micrograph images were quantified using CellProfiler [43]. Nuclei were identified from DAPI images using the “IdentifyPrimaryObjects” module. Cytoplasmic regions were derived by expanding nuclei (“ExpandOrShrinkObjects” module) and subtracting the original nuclei (“IdentifyTertiaryObjects” module), nuclear regions were derived by shrinking original nuclei (“ExpandOrShrinkObjects” module). YAP intensity (488 channel) was measured in cytoplasm and nuclei using the “MeasureObjectIntensity” module. Cell area was calculated from phase contrast images using ImageJ[44].

Statistical Analysis: After testing data sets for normality, statistical analysis was performed using two-way ANOVA, one-way ANOVA with Student-Newman-Keul’s post-test or where appropriate a paired Student’s t-test, as indicated. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$.

RESULTS

Adenosine A2B activation and elevated cAMP inhibits EPAC1 expression in cardiac fibroblasts

Activation of the adenosine A2B-receptor elevates intracellular cAMP levels and has anti-fibrotic effects in cardiac fibroblasts [29]. We initially stimulated cardiac fibroblasts with the adenosine A2B receptor agonist BAY60-6583 (at 5 μ g/mL as previously described [45]) and quantified EPAC1 mRNA and protein levels at early timepoints (i.e. less than 8 hours) to identify direct effects on EPAC1 levels and avoid confounding effects of secondary signalling mechanisms. We focused on the regulation of EPAC1 as we were unable to detect EPAC2 protein expression in cardiac fibroblasts by Western blotting (Supplement Figure 1), consistent with previous observations [33]. Stimulation with BAY60-6583 significantly reduced EPAC1 mRNA levels after 2 and 8 hours (Figure 1A). Levels of EPAC1 pre-spliced RNA, a surrogate measure of EPAC1 transcriptional rate[46], were similarly reduced at 2 and 8 hours (Figure 1A). Levels of the housekeeping gene 36B4 were unaffected. BAY60-6583 also significantly reduced levels of EPAC1 protein after 2, 4, 6 and 8 hours, with maximal inhibition at the 8-hour time-point (Figure 1B). Similar down regulation of EPAC1 mRNA was detected in human cardiac fibroblasts, stimulated with BAY-60-6583 (Supplementary Figure 2A). We next tested if elevating intracellular cAMP levels with the adenylate cyclase agonist forskolin (at 25 μ M as previously described [42]) or the cAMP analogue, dibutyryl-cAMP (at 200 μ M as previously described [41]) also inhibited EPAC1 expression. Stimulation with forskolin significantly inhibited EPAC1 mRNA and pre-spliced EPAC1 RNA at 2 and 8 hours without affecting 36B4 mRNA levels (Figure 1C). Forskolin stimulation also resulted in time dependent inhibition of EPAC1 protein levels, with maximal inhibition after 8 hours (Figure 1D). Stimulation of human CF with forskolin also resulted in the downregulation of the protein and mRNA levels of EPAC1 after 8 hours (Supplementary Figure 2B). In a similar manner, stimulation with dibutyryl-cAMP also significantly reduced EPAC1 mRNA and pre-spliced EPAC1 mRNA at 2 and 8 hours (Figure 1E) and EPAC1 protein levels (Figure 1F). Elevated cAMP can activate both PKA and EPAC. We therefore tested whether PKA or EPAC activity was involved in inhibition of EPAC1 expression in cardiac fibroblasts. We initially used the PKA and EPAC selective pharmacological inhibitors H89 and ESI-09{Almahariq, 2013 #3608}. Treatment with H89 or ESI-09 significantly reversed the forskolin-induced reduction in EPAC1 mRNA and pre-spliced EPAC1 mRNA levels (Figure 2A), suggesting involvement of both PKA and EPAC activity in the regulation of EPAC1 mRNA levels. To further test the role of PKA and EPAC activity, we stimulated cells with the PKA selective agonist 6-BNZ-cAMP-AM or the non-cyclic EPAC selective agonist I-942 [47]. Stimulation with 6-BNZ-cAMP-AM or I-942 alone resulted in a significant down regulation of

EPAC1 mRNA and pre-spliced EPAC mRNA without affecting levels of 36B4 (Figure 2B). A combination of I-942 plus 6-BNZ-cAMP-AM acted additively to suppress EPAC1 mRNA and pre-spliced RNA levels. To further test the role of EPAC activation in the regulation of EPAC1 mRNA levels, we stimulated cells with the cell permeable EPAC selective cAMP analogue 8-pCPT-2'-O-Me-cAMP-AM (abbreviated to 8-CPT-AM) alone or in combination with the PKA selective agonist 6-BNZ-cAMP-AM. Stimulation with 8-pCPT-2'-O-Me-cAMP-AM or 6-BNZ-cAMP-AM alone significantly down regulated EPAC1 mRNA and pre-spliced EPAC mRNA without affecting levels of 36B4 (Figure 2C). A combination of 6-BNZ-cAMP-AM plus 8-pCPT-2'-O-Me-cAMP-AM acted additively to suppress EPAC1 mRNA and pre-spliced RNA. EPAC1 protein levels were significantly inhibited in response to 6-BNZ-cAMP-AM or a combination of 6-BNZ-cAMP plus 8-pCPT-2'-O-Me-cAMP-AM but not by 8-pCPT-2'-O-Me-cAMP-AM alone (Figure 2D).

Inhibition of EPAC1 expression by cAMP is associated with actin-cytoskeleton remodelling and changes in cell morphology

Elevated levels of cAMP have previously been shown to modulate cell morphology and actin polymerisation in several cell types {Bond, 2008 #1738; Duggirala, 2015 #3210;Hewer, 2011 #1841; Kimura, 2016 #3418; Wu, 2006 #1597; Pelletier, 2005 #2641}, and this has been functionally linked to cAMP-induced changes in cell behaviour. This typically occurs as a result of cAMP-mediated inhibition of RhoGTPase activity [48]. We noted that inhibition of EPAC1 expression by cAMP-elevating stimuli in cardiac fibroblasts was associated with a reduction in cell spreading (Figure 3B) and a change in cell morphology (Figure 3D and F), characterised by acquisition of a condensed stellate-like morphology and loss of F-actin-stress fibres (Figure 3E), implying impaired actin-polymerisation. We asked if changes in cell morphology and actin polymerisation were mechanistically linked to regulation of EPAC1 levels. Forskolin or BAY60-6583 stimulation of cardiac fibroblasts induced a significant reduction in the F-actin:G-actin ratio (Supplement Figure 3A), confirming reduced actin polymerisation in response to elevated cAMP signalling. This was also associated with significant reductions in phosphorylation of the ROCK substrate, MYPT (Supplement Figure 3B-D), indicating inhibition of RhoA-ROCK signalling. By contrast, cAMP-elevating stimuli did not induce any detectable morphological change, reduction in cell area, loss of F-actin stress fibres or a reduction in EPAC1 mRNA expression in the myocyte H9C2 cell line (Figure 3A-E). In cardiac fibroblasts, stimulation with the PKA selective agonist 6-BNZ-cAMP-AM alone induced a detectable change in morphology that was further enhanced by co-stimulation with 8-pCPT-2'-O-Me-cAMP-AM (Figure 3F) or I-942 (Supplement Figure 4).

Taken together, these data indicate that cAMP-induced down-regulation of EPAC1 is associated with disruption of actin-polymerisation and changes in cell morphology.

To further test the association between cell morphology and EPAC1 expression we tested if washout of the cAMP stimulus would lead to restoration of cell morphology and EPAC1 mRNA expression levels. Forskolin stimulation for 2 hours, 8 hours or 24 hours significantly inhibited EPAC1 mRNA expression (Supplement Figure 5A) and induced a characteristic stellate morphology (Supplement Figure 5B). Importantly, levels of EPAC1 mRNA levels after a 2-hour forskolin stimulation followed by a 6-hour or 22-hour washout period were not significantly different from control levels, indicating that removal of the cAMP stimulus lead to a rapid restoration of EPAC1 expression. Furthermore, washout of forskolin resulted in a rapid reversal of the forskolin-induced morphological change (Supplement Figure 5B).

We then asked whether actin polymerisation is required for maximal EPAC1 expression in cardiac fibroblast. We disrupted actin polymerisation using the Rho kinase (ROCK) inhibitor Y-27632 and the actin binding drugs latrunculin-B (Lat-B) and cytochalasin-D (Cyto-D). Treatment of cardiac fibroblasts with either Y-27632, Lat-B or Cyto-D induced stellate cell morphology and a loss of F-actin stress fibres (Figure 4A). Treatment with Y-27632, Lat-B or Cyto-D also significantly reduced cell spreading (Figure 4B) and significantly inhibited EPAC1 mRNA and pre-spliced RNA levels, without affecting mRNA levels of the housekeeping gene 36B4 (Figure 4C). Y-27632, Lat-B or Cyto-D stimulation also significantly reduced levels of EPAC1 protein (Figure 4D).

cAMP-induced down regulation of EPAC1 impairs EPAC downstream signalling and cAMP-induced morphological change.

We next asked if down regulation of EPAC1 expression in response to elevated cAMP resulted in impaired signalling downstream of EPAC1 and impaired cAMP-induced cellular responses. Cells were pre-treated with forskolin for 24 hours to down regulate EPAC1 expression, which was confirmed by Western blotting (Supplement Figure 6A). Cells were then acutely stimulated with either 8-pCPT-2'-O-Me-cAMP-AM or forskolin for 30 mins and activation of Rap1 assayed. In non-pre-treated cells, acute stimulation with either 8-pCPT-2'-O-Me-cAMP-AM or forskolin resulted in an increase in Rap1_{GTP} (active) levels (Supplement Figure 6C and D). However, in cells pre-treated with forskolin for 24 hours, acute stimulation with 8-pCPT-2'-O-Me-cAMP-AM or forskolin did not result in an increase in Rap1_{GTP}. Furthermore, the morphological change (quantified as a reduction in cell spreading) induced by either forskolin or a combination of 8-pCPT-2'-O-Me-cAMP-AM plus 6-BNZ-cAMP was significantly reduced in cells pre-treated with

forskolin (Supplement Figure 7 and 8). These data indicate that cAMP-induced down regulation of EPAC1 impairs EPAC1 down-stream signalling and cAMP-induced morphological changes.

cAMP mediated inhibition of YAP-TEAD decreases EPAC1 promoter activity

We transiently transfected cardiac fibroblasts with a secreted nano-luciferase reporter gene driven by the human EPAC1 proximal promoter region (EPAC1-NLUC) to determine if elevated cAMP and actin-cytoskeleton disruption controls EPAC1 levels at the level of transcription. Forskolin stimulation of EPAC1-NLUC transfected cells resulted in a significant inhibition of promoter activity but not of a control reporter gene driven by the TNT-minimal promoter region (Figure 5A). Furthermore, disruption of actin-cytoskeleton polymerisation using Y-27632, latrunculin-B or cytochalasin-D also significantly inhibited EPAC1 promoter activity (Figure 5B). To begin to identify potential transcription factors that might mediate this regulation, we constructed a series of EPAC1 promoter truncations (-800, -600, -400 and -200 bp). Forskolin stimulation of cardiac fibroblasts transfected with these promoter truncations demonstrated that all promoter fragments were susceptible to inhibition by forskolin (Figure 5C), implying that a binding element for a cAMP-sensitive transcription factor mediating repression of EPAC1 transcription is located within the proximal 200 bp of the EPAC1 promoter. Sequence analysis of this region identified a conserved binding element for the transcription factor (TEAD). Several reports have previously linked actin-cytoskeleton organisation to regulation of TEAD transcription factor activity [38, 40, 49, 50]. We therefore tested if cAMP-dependent regulation of EPAC1 expression in cardiac fibroblasts was mediated by inhibition of TEAD activity. We initially asked if elevated cAMP or actin-cytoskeleton disruption inhibited TEAD activity and TEAD target gene expression. Stimulation of cardiac fibroblasts transfected with a synthetic TEAD reporter gene with cAMP-elevating stimuli (forskolin, dibutyryl-cAMP or BAY60-6583) resulted in a significant inhibition of TEAD-dependent reporter gene activity, without significantly affecting activity of a TEAD-independent minimal TNT-promoter (Figure 5D). Likewise, disruption of actin-cytoskeleton polymerisation with latrunculin-B, cytochalasin-D or Y-27632 also resulted in a significant inhibition of TEAD-dependent transcription (Figure 5E). Furthermore, forskolin stimulation resulted in significant inhibition of mRNA levels of the classical TEAD-target genes, *CTGF* and *CCNI*, consistent with cAMP-mediated inhibition of TEAD activity (Figure 5F). TEAD activity is typically controlled by its transcriptional co-factor YES-associated protein (YAP) [51, 52]. Immunofluorescent staining of forskolin stimulated cardiac fibroblasts detected a significant reduction in nuclear and cytoplasmic levels of YAP protein (Figure 5G). Furthermore, Western blot analysis of total cell lysates confirmed a significant and rapid downregulation of total YAP

protein levels (Figure 5H and I) and an increase in the level of YAP phosphorylation at serine 172 (Figure 5H and J) and serine 397 (Figure 5H and K). These posttranslational modifications have previously been associated with YAP nuclear export and degradation [52, 53]. Taken together, these data demonstrate that elevated cAMP and cytoskeleton disruption inhibit EPAC1 promoter activity and TEAD activity in cardiac fibroblasts.

To investigate if the consensus TEAD element in the EPAC1 proximal promoter was functionally important for EPAC1 expression we tested if this promoter element bound TEAD1 protein and if it was required for maximal promoter activity. Electromobility shift assay (EMSA) analysis demonstrated that nuclear extracts from Myc-TEAD1 transfected fibroblasts but not control transfected cells produced a band of reduced mobility when incubated with a biotinylated DNA probe containing the wild-type EPAC1 promoter TEAD element, indicating that TEAD1 protein is able to bind the consensus TEAD element in the EPAC1 promoter (Figure 6A). Furthermore, this band was lost when a 3 base-pair mutation was introduced into the TEAD element, indicating sequence-specific TEAD1 interaction. In the context of a reporter gene assay, the introduction of this 3 bp mutation significantly inhibited EPAC1 promoter activity (Figure 6B), indicating that this TEAD element is required for maximal EPAC1 promoter activity in cardiac fibroblasts. To further confirm this, we used siRNA-mediated silencing of the TEAD co-factors, YAP and TAZ. Transfection of cardiac fibroblasts with siRNA targeting YAP and TAZ resulted in a strong down regulation of YAP and TAZ protein expression without affecting levels of GAPDH or β -actin protein (Figure 6C). A similar down regulation of YAP and TAZ mRNA but not the housekeeping gene 36B4 (Figure 6F) was also detected after YAP and TAZ silencing (Figure 6D). YAP and TAZ silencing also significantly inhibited TEAD-reporter gene activity (Figure 6D), consistent with downregulation of these TEAD co-factors. Importantly, YAP and TAZ silencing also significantly inhibited EPAC1 promoter activity (Figure 6E). Furthermore, YAP and TAZ silencing also reduced mRNA levels of the classical TEAD-target gene *CCN1* as well as significantly reducing EPAC1 mRNA and EPAC pre-spliced mRNA levels without affecting the mRNA levels of the housekeeping gene *36B4* (Figure 6F). To test the role of TEAD further, we transfected cardiac fibroblasts with a dominant-negative TEAD1 expression vector that expresses the TEAD1 DNA binding domain fused to the *Drosophila* engrailed transcriptional repressor domain (TEAD1-Eng). Expression of TEAD1-Eng significantly inhibited TEAD reporter gene activity without significantly affecting activity of a TEAD-independent minimal promoter reporter gene (Figure 6G), consistent with efficient and specific inhibition of TEAD activity. Importantly, expression of TEAD1-Eng also significantly inhibited EPAC1 reporter gene activity, without affecting activity of a minimal promoter reporter gene (Figure 6H).

The formation of a YAP-TEAD complex is a critical step in activation of TEAD-dependent transcription. We therefore used two structurally distinct pharmacological inhibitors of the YAP-TEAD complex, namely Verteporfin [54] and CPD3.1 [55], to further test the role of YAP-TEAD in EPAC1 gene expression. Treatment with Verteporfin or CPD3.1 significantly inhibited TEAD-NLUC activity (Figure 7A). Treatment with Verteporfin or CPD3.1 also significantly inhibited EPAC1 promoter activity (Figure 7B) and reduced the mRNA levels of the classical TEAD-target genes *CCNI* and *CTGF* as well as inhibiting levels of EPAC1 mRNA and pre-spliced mRNA (Figure 7C and D). Levels of the TEAD-independent housekeeping genes 36B4 or TBP were unaffected by either Verteporfin or CPD3.1 (Figure 7 C and D). Taken together, these data demonstrate that YAP/TAZ-TEAD activity is required for maximal EPAC1 gene expression in cardiac fibroblasts.

YAP and TAZ are required but insufficient for EPAC1 mRNA expression

We used adenovirus mediated over expression of constitutively-active mutants of YAP (YAP_{S127A}) and TAZ (TAZ_{55A}) to test if YAP or TAZ activation are sufficient for EPAC1 expression and can reverse the inhibitory effects of elevated cAMP. Adenovirus-mediated expression of YAP_{S127A} completely reversed forskolin-mediated inhibition of TEAD-reporter activity, confirming efficient reversal of cAMP-mediated inhibition of TEAD activity (Figure 8A). Over expression of YAP_{S127A} also completely reversed the forskolin-mediated inhibition of EPAC1 promoter activity (Figure 8B), consistent with our data demonstrating a requirement for YAP-TEAD activity for maximal EPAC1 expression. We therefore tested if YAP_{S127A} over expression could also rescue mRNA levels of *CCNI* or *EPAC1* after forskolin stimulation. Forskolin stimulation of cells infected with a control adenovirus lacking a transgene resulted in significant reduction in the mRNA levels of *CCNI* (Figure 8C). Moreover, this inhibition was completely reversed by expression of either YAP_{S127A} or TAZ_{55A}, consistent with our data on rescue of TEAD-dependent reporter gene activity (Figure 8C). Forskolin also decreased EPAC1 mRNA levels in control virus expressing cells (Figure 8D). However, expression of YAP_{S127A} or TAZ_{55A} did not significantly rescue forskolin inhibition of *EPAC1* mRNA levels (Figure 8D). The results implied that YAP/TAZ-TEAD activity alone is insufficient for EPAC1 mRNA expression in cardiac fibroblasts, despite the knockdown and dominant negative data in Figures 6 and 7 showing that YAP/TAZ-TEAD activity is required. This suggests the involvement of at least one additional mechanism that is required for expression of the endogenous EPAC1 gene that is not required for expression of the EPAC1 reporter gene activity.

HDAC1/3-mediated deacetylation of histone-3 lysine 27 contributes to cAMP-mediated inhibition of EPAC1 expression

Plasmid-based reporter gene vectors are not typically packaged with histone proteins into chromatin and are largely regulated by transcription factor activity alone. However, endogenous genes are dependent on both transcription factor activity and histone-associated epigenetic regulation. We therefore considered the possibility of epigenetic control in cAMP-mediated repression of EPAC1 expression. Since acetylation of histone-3 at lysine-27 is associated with an open chromatin conformation and active gene expression, we specifically asked whether epigenetic regulation of histone3 lysine-27 acetylation is necessary, in addition to YAP-TEAD activity, to promote EPAC1 mRNA expression and whether deacetylation mediates the repression of EPAC1 mRNA expression in response to elevated cAMP. Furthermore, we investigated whether a requirement of histone-3 lysine-27 (H3K27) acetylation explains inability of YAP or TAZ over expression to rescue EPAC1 mRNA levels in forskolin stimulated cells. We used chromatin immunoprecipitation (ChIP) to quantify the effects of cAMP elevation on H3K27 acetylation at the proximal EPAC1 promoter. H3K27 acetylation was detected at the EPAC1 proximal promoter region in cultures of cardiac fibroblasts. Moreover, stimulation with forskolin resulted in a significant reduction in H3K27 acetylation levels at this region (Figure 9A). In contrast, levels of H3K27 acetylation at the proximal promoter of HAS1, a cAMP-stimulated CREB target gene, were significantly increased in response to forskolin stimulation, indicating that forskolin induced changes in H3K27 acetylation are region specific and do not simply reflect global changes H3K27-acetylation levels. Importantly, the forskolin-induced reduction of H3K27-acetylation at the EPAC1 promoter was partially but significantly reversed by pre-treatment with the HDAC1/3-selective HDAC inhibitor MS-275, which was not the case for CREB-target gene, HAS1, indicating region specific changes in H3K27-acetylation mediated by HDAC1/3 (Figure 9B).

We next asked whether HDAC1/3 inhibition with MS-275 would synergise with YAP_{S127A} overexpression to rescue forskolin inhibition of EPAC1 mRNA. In control virus infected cells forskolin significantly inhibited EPAC1 mRNA levels (Figure 9C), which were not rescued by YAP_{S127A}, in agreement with the data in Figure 8. By contrast, MS-275 partially antagonised the ability of forskolin to reduce EPAC1 mRNA levels. Importantly, however, a combination of MS-275 and YAP_{S127A} expression completely prevented forskolin-mediated inhibition of EPAC1 mRNA levels, whereas expression of the housekeeping gene 36B4 remained unaffected by any of the treatments (Figure 9D). Hence H3K27 acetylation appeared to facilitate TEAD dependent

transcription of EPAC1 and cAMP elevation triggered H3K27-deacetylation as well as blocking TEAD activity.

DISCUSSION

The ability of cAMP to stimulate EPAC activity is well established[56] [41] [57] [58]. However, relatively little is known about how EPAC gene expression is regulated. In this study we investigated the regulation of EPAC1 gene expression in cardiac fibroblast under normal culture conditions and in response to cAMP-elevating stimuli. We showed that EPAC1 mRNA and protein expression are supported by TEAD-dependent transcription and that H3K27 acetylation is required for TEAD activity at the EPAC proximal promoter. Furthermore, we demonstrate that elevated intracellular cAMP rapidly represses the expression of EPAC1 mRNA and protein levels by antagonising both processes. The first involves cAMP-induced actin-cytoskeleton depolymerisation and subsequent inactivation of YAP/TAZ-TEAD transcription factor activity. The second involves cAMP-mediated deacetylation of H3K27 at the EPAC1 proximal promoter region. Repression of EPAC1 expression via these mechanisms attenuates EPAC1 signalling and EPAC1-dependent morphological changes in response to subsequent cAMP-elevating stimuli. Together, these mechanisms constitute a newly discovered mechanism of crosstalk that represses EPAC1 expression in response to cAMP elevating stimuli and is important in controlling cellular responses to cAMP elevating stimuli (Figure 10).

In detail, cAMP elevation in cardiac fibroblasts using either forskolin, cAMP analogues or physiological agonists of the adenosine A2B receptor rapidly represses EPAC1 expression in cardiac fibroblasts. Selective activation of either EPAC or PKA alone inhibited EPAC1 mRNA expression, with simultaneous activation of PKA and EPAC acting additively. Interestingly, PKA activation was more effective than EPAC activation at down regulating EPAC1 expression, suggesting a dominant role for PKA-mediated negative crosstalk in controlling EPAC1 expression. Negative crosstalk between PKA and EPAC have been reported previously. For example, PKA can antagonise EPAC induced phosphorylation of AKT [59]. PKA can reduce the ability of the EPAC effector protein Rap1 to regulate angiogenesis [60]. PKA can also regulate cAMP-dependent activation of EPAC by controlling the availability of cAMP via phosphodiesterases [61]. Our new data now demonstrate that PKA and EPAC-activation both play an important role in regulating EPAC1 gene expression in cardiac fibroblasts.

Repression of EPAC1 expression in cardiac fibroblasts in response to elevated cAMP is associated with acquisition of a condensed ‘stellate’ shaped cell morphology characterised by a condensed

cytoplasm, extended membrane protrusion and loss of F-actin stress fibres. Similar cAMP-induced morphological changes and inhibition of actin-polymerisation have been described in some other cell types, including smooth muscle cells[41, 42, 62, 63], astrocytes [64] and mesenchymal stem cells [65]. Interestingly, in cardiac fibroblasts, the magnitude of the stellate morphology and reduction in actin-cytoskeleton polymerisation induced by activation of PKA and EPAC mirrors the repression of EPAC1 gene expression. However, these cAMP-induced stellate morphological changes and inhibition of actin polymerisation are cell-type specific. Although elevated cAMP induces rapid and pronounced actin-cytoskeleton disruption in cardiac fibroblasts and smooth muscle cells[41, 48, 66], this does not occur in endothelial cells, where cAMP signalling promotes cortical actin polymerisation via PKA and EPAC activation [6, 67-69]. We have shown that cAMP signalling does not induce morphological changes or inhibition of actin polymerisation in the H9C2 cardiac myocyte cell line. Importantly, EPAC1 expression is not repressed by cAMP-elevating stimuli in these cells, implying that repression of EPAC1 expression is, at least in part, mediated by these changes in the actin-cytoskeleton polymerisation status. Consistent with this, we demonstrated that disruption of actin-polymerisation, using the ROCK inhibitor Y-26732, or directly with the actin-binding drugs cytochalasin-D or latrunculin-B, also repress EPAC1 expression in cardiac fibroblasts. To our knowledge this is the first time that EPAC1 expression has been linked to actin-cytoskeleton remodelling, although previous reports have linked EPAC1 localisation and function to actin organisation. For example, EPAC1 interaction with the actin binding ERM (Ezrin, Radixin and Moesin) proteins has been implicated in localisation of EPAC1 to the plasma membrane cortical actin, where it participates in cAMP-dependent regulation of cell proliferation and cell spreading [70-72]. Our new data now establish the existence of a novel negative crosstalk mechanism where PKA cooperates with EPAC activity to induce actin-cytoskeleton remodelling, which contributes to repression of EPAC1 gene expression.

Several signals have been linked to modulation of EPAC1 expression. TGF- β stimulation can have stimulatory or repressive effects on EPAC1 expression in cardiac fibroblasts or myofibroblasts respectively [5]. Prostaglandin E₂ stimulation reduces EPAC1 protein expression in carbon tetrachloride-induced liver fibrosis in mice [73]. β -adrenoceptor (AR) receptor activation with isoproterenol increases EPAC1 expression in atrial fibroblasts [14]. However, the underlying mechanisms that control EPAC1 gene expression in response to physiological signals is unknown. Here we describe a feedback mechanism that represses EPAC1 gene transcription in response to elevated cAMP. For example, we show that levels of pre-spliced EPAC mRNA, a surrogate measure of transcriptional rate [46], are rapidly down regulated by cAMP-elevating stimuli. Furthermore, the activity of the proximal EPAC1 promoter region is also repressed by these

stimuli, consistent with transcriptional mode of repression. Analysis of the EPAC1 proximal promoter region identified a conserved binding element for the TEAD transcription factors. Importantly, TEAD transcription factor activity is known to be dependent on actin-cytoskeleton organisation and integrity [38] [74], consistent with our data showing that maximal EPAC1 expression is also dependent on actin-cytoskeleton. We show that elevated cAMP represses TEAD activity in cardiac fibroblasts and this is associated with increased phosphorylation of the TEAD co-factor YAP and a reduced levels of YAP protein in the nucleus and cytoplasm. We demonstrate that maximal EPAC1 expression is dependent on YAP/TAZ-mediated TEAD activity. For example, inhibition of TEAD activity using silencing of the TEAD co-factors YAP and TAZ, overexpression of a dominant-negative TEAD mutant or inhibition of TEAD activity using two chemically distinct pharmacological inhibitors all significantly inhibited EPAC1 transcription. To our knowledge, this is the first time that EPAC1 expression has been linked to cytoskeleton integrity and YAP/TAZ-TEAD activity.

Although we demonstrate a clear requirement for YAP/TAZ-TEAD activity for EPAC1 expression, our data also demonstrate that activation of this mechanism in isolation, via expression of constitutively active YAP or TAZ mutants, is insufficient for expression of EPAC1 mRNA, highlighting the involvement of additional mechanisms. While expression of constitutively active YAP or TAZ were able to completely reverse cAMP-dependent repression of the EPAC1 promoter driven reporter gene activity, they were not able to reverse the inhibition of EPAC1 mRNA levels. Since plasmid-based reporter gene vectors are not typically packaged with histone proteins into chromatin, we reasoned that histone-associated epigenetic regulation was also involved in cAMP-mediated repression of EPAC1 expression. Consistent with this, we detected a significant decrease in histone-3 lysine-27 acetylation (H3K27Ac), an epigenetic modification associated with active transcription[75], at the proximal EPAC1 promoter region in cardiac fibroblasts stimulated with forskolin. Our data suggest that this loss of H3K27Ac was mediated by HDAC1 or HDCA3, since loss of H3K27Ac at the EPAC1 promoter in response to forskolin stimulation was reversed by the HDAC1/3 selective inhibitor MS-275. Importantly, our data demonstrates that this epigenetic modification acts cooperatively with inhibition of YAP/TAZ-TEAD to mediated cAMP-dependent repression of EPAC1 expression. For example, HDAC inhibition or expression of constitutively active YAP/TAZ alone only partially rescued EPAC1 mRNA expression in forskolin-stimulated cells, whereas HDAC inhibition together with constitutively-active YAP/TAZ fully reverse the inhibitory effects of forskolin.

Taken together, our data demonstrate that inhibition of YAP/TAZ-TEAD and HDAC1/3 - mediated H3K27 deacetylation underlies the repression of EPAC1 expression in cardiac

fibroblasts in response to cAMP-elevating stimuli. We suggest that this mechanism represents a novel mechanism of cross talk that controls EPAC1 signalling. Such loops that feed an inverted output signal back to the input are found in nearly all known signalling pathways, serving to maintain normal physiological signalling homeostasis and allowing for adaptive cellular responses to a persistent signal. Down regulation of EPAC1 expression via the control loop we describe here is likely to be important in the temporal regulation of EPAC1 signalling, generating transient signals responses in prolonged cAMP elevation. Selective down regulation of EPAC1 but not PKA catalytic subunits suggests that subsequent cAMP signalling may also be adapted towards PKA-specific responses.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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FIGURES

Figure 1

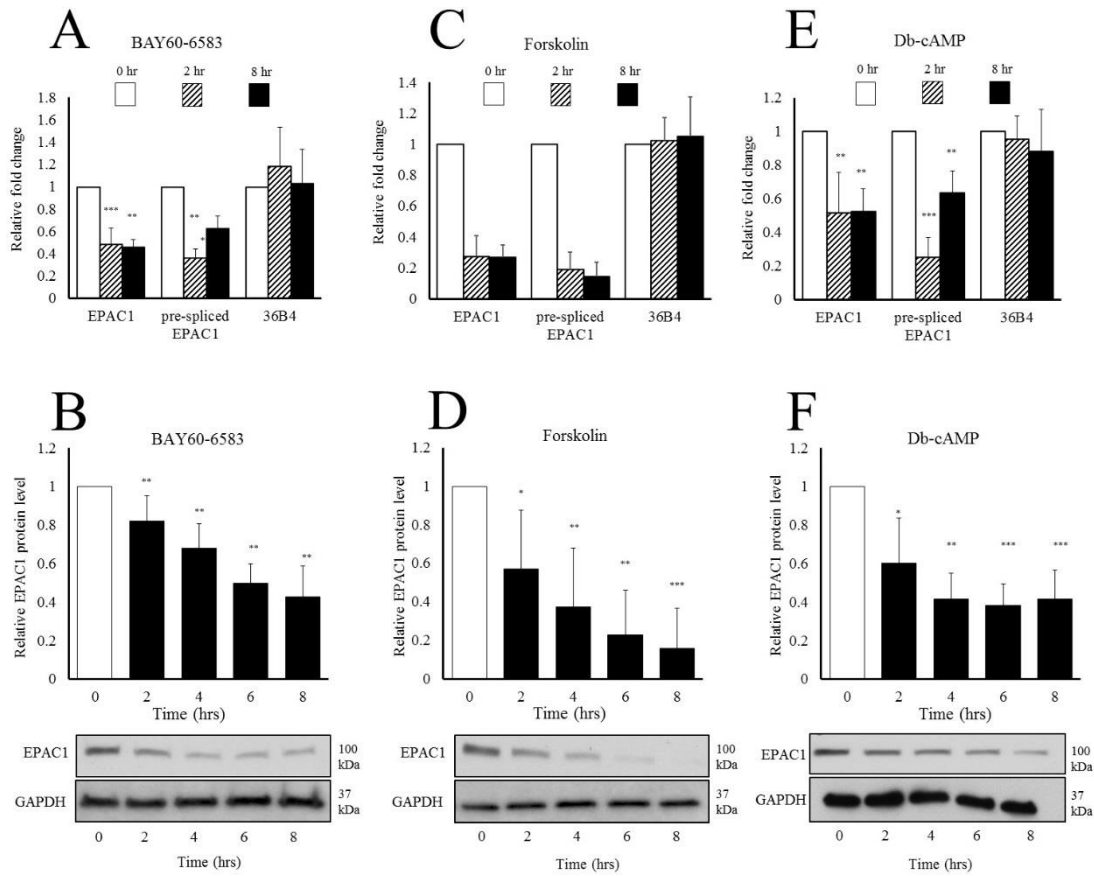


Figure 1: Adenosine A2B activation and elevated cAMP inhibits EPAC1 expression in cardiac fibroblasts

Serum-starved CF were stimulated with BAY60-6583 (5 μ g/mL; A-B), forskolin (25 μ M; C-D) and dibutyl-cAMP (200 μ M; E-F) for 2 to 8 hours. Total RNA was extracted and analysed by qRT-PCR for fully spliced and pre-spliced EPAC1 and 36B4 mRNA (A, C and E). Total cell lysates were analysed for the levels of EPAC1 and GAPDH protein by Western blotting and densitometric analysis (B, D, F). ANOVA with Student Newman Keul's post-test. Data are expressed as mean \pm SD (n = 3). *, ** and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively.

Figure 2

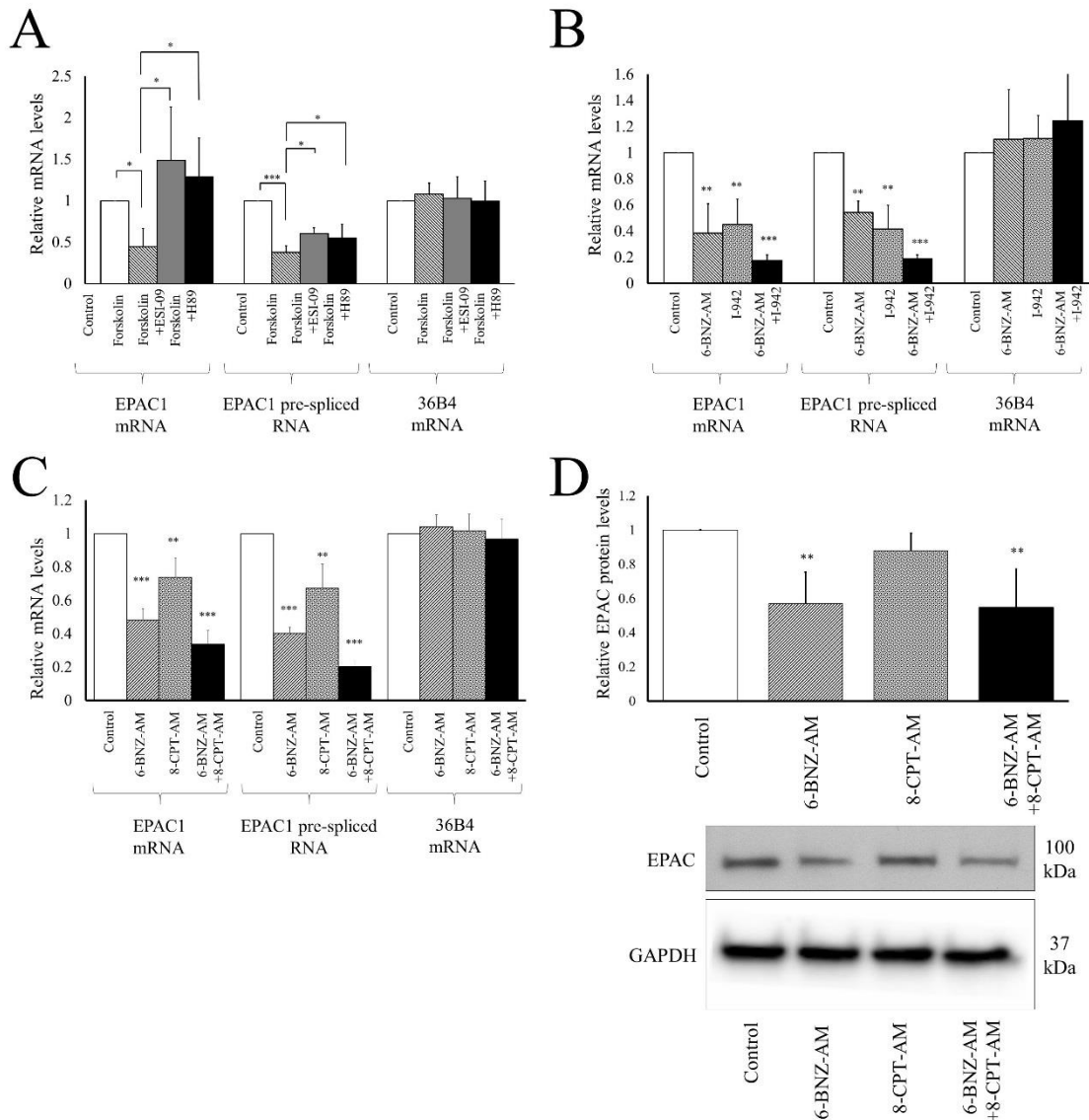


Figure 2: PKA and EPAC activity co-ordinately regulate EPAC1 expression in cardiac fibroblasts

Serum-starved CF were stimulated forskolin (25 μ M) alone or in combination with either H89 (10 μ M) or ESI-09 (10 μ M), as indicated, for two hours (A) or with 6-BNZ-cAMP-AM (6-BNZ-AM; 20 μ M), I-942 (100 μ M) or 8-pCPT-2'-O-Me-cAMP-AM (8-CPT-AM, 20 μ M) as indicated, for 8 hours (B). Total RNA was extracted and analysed for EPAC1 mRNA, pre-spliced EPAC1 mRNA and 36B4 mRNA by qRT-PCR (A; n=5, B and C; n=3). Total cell lysates were analysed for EPAC1 and GAPDH protein levels by Western blotting and densitometric analysis (D; n=4). Data are expressed as mean \pm SD and analysed by ANOVA with Student Newman Keul's post-test. (n = 3). *, ** and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively.

Figure 3

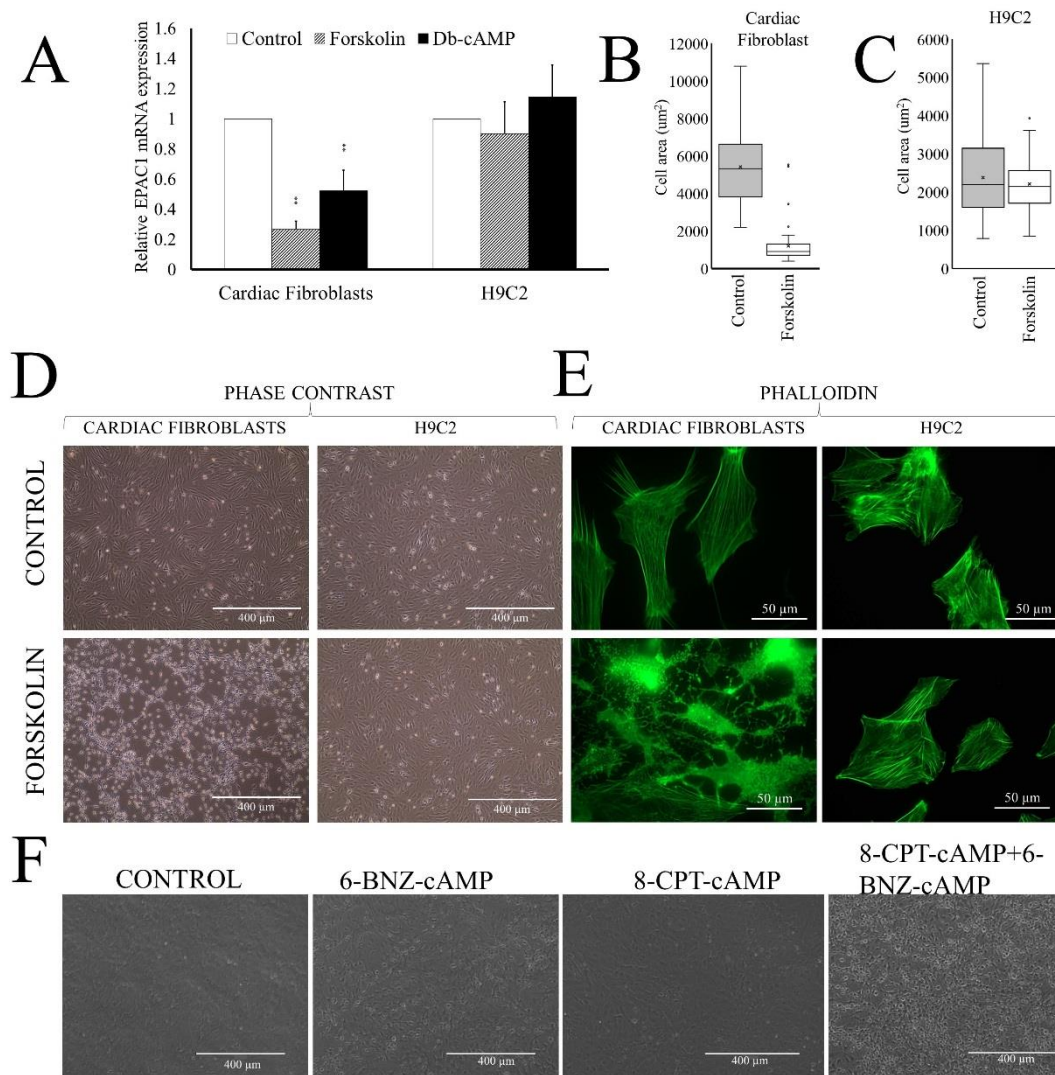


Figure 3: Inhibition of EPAC1 expression by cAMP is associated with actin cytoskeleton remodelling and changes in cell morphology

Serum-starved CF and H9C2 cells were stimulated with forskolin (25 µM) or dibutyryl-cAMP (200 µM) for 8 hours, as indicated. Total RNA was extracted and analysed for EPAC1 mRNA by qRT-PCR (A). Cell spreading (B and C) and cell morphology (D) was analysed by phase contrast microscopy. F-actin stress fibres were detected by phalloidin staining (E). Serum-starved CF were stimulated with 6-BNZ-cAMP-AM (6-BNZ-AM, 20 µM) and 8-pCPT-2'-O-Me-cAMP-AM (8-CPT-AM, 20 µM), alone or in combination and cell morphology assessed by phase contrast microscopy (F). Data are expressed as mean \pm SD and analysed by ANOVA with Student Newman Keul's post-test. (n = 3). ** indicates $p < 0.01$.

Figure 4

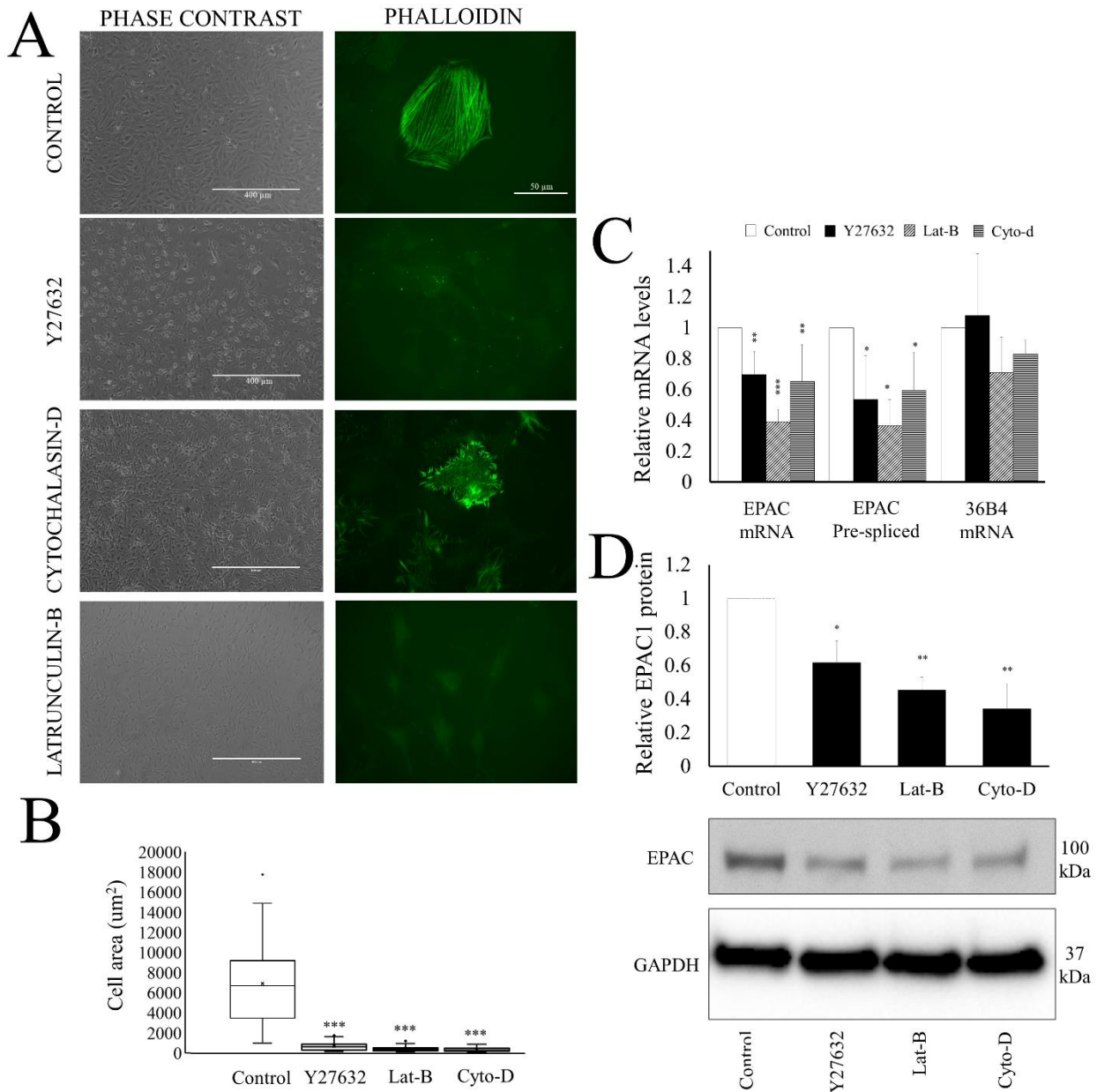


Figure 4: Actin cytoskeleton disruption inhibits EPAC1 expression

Serum-starved CF were stimulated with 10 μ M Y-27632, 2 μ M cytochalasin-D (Cyto-D) or 0.5 μ g/mL latrunculin-B (Lat-B) for 8 hours. Cell morphology and F-actin stress fibres were analysed by phase contrast microscopy and phalloidin staining, respectively (A). Cell area was quantified by image analysis of phase contrast images (B). Total RNA was extracted and analysed for EPAC1 mRNA, pre-spliced EPAC1 mRNA and 36B4 mRNA by qRT-PCR (C; n=3). Total cell lysates were analysed for EPAC1 and GAPDH protein levels by Western blotting and densitometric analysis (D; n=5). Data are expressed as mean \pm SD and analysed by ANOVA with Student Newman Keul's post-test. *, ** and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively.

Figure 5

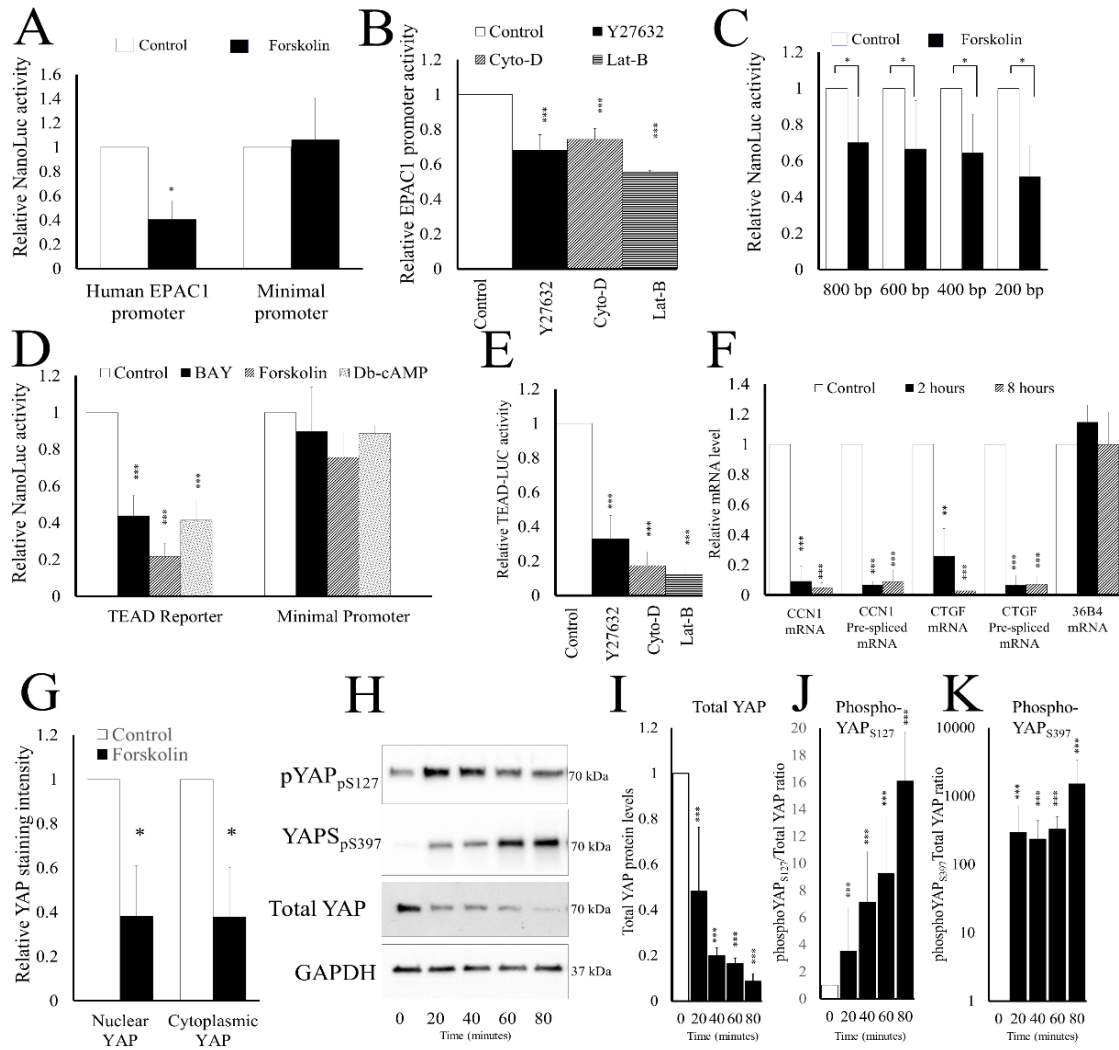


Figure 5: Elevated cAMP and inhibition of actin polymerisation reduces EPAC1 promoter activity and TEAD activity in cardiac fibroblasts

Cardiac fibroblasts were transfected with EPAC1-NLUC reporter (A and B) and stimulated with 25 μ M forskolin, 10 μ M Y-27632, 2 μ M Cyto-D or 0.5 μ g/mL Lat-B for 8 hours. Cells were transfected with a NLUC reporter vector controlled by the indicated EPAC1 promoter truncations or a minimal promoter, as indicated, and NLUC activity assayed 24 hours later (C). Cells were transfected with a TEAD-LUC reporter or minimal promoter-LUC reporter vector as indicated and stimulated with 5 μ g/mL BAY65-6085, 25 μ M forskolin, 200 μ M db-cAMP and luciferase activity quantified after 8 hours (D). Cardiac fibroblasts were transfected with a TEAD-LUC reporter vector and stimulated with 10 μ M Y-27632, 2 μ M Cyto-D and 0.5 μ g/mL Lat-B and luciferase activity was quantified after 8 hours (E). Cardiac fibroblasts were stimulated with 25 μ M forskolin for 2 and 8 hours. Total RNA was extracted and analysed for CCN1 mRNA, pre-spliced CCN1 RNA, CTGF mRNA, pre-spliced CTGF RNA and 36B4 mRNA levels. CF were stimulated with 25 μ M forskolin for 2 hours (G) or indicated times (H-K). Nuclear and cytoplasmic YAP levels were quantified by image analysis of immunofluorescent micrographs (G). Total cellular YAP, phospho-YAP_{S127}, phospho-YAP_{S397} were quantified by Western blotting (H) and densitometric analysis (I-K). Data are expressed as mean \pm SD and analysed by

ANOVA with Student Newman Keul's post-test. *, ** and *** indicate $p<0.05$, $p<0.01$ and $p<0.001$ respectively.

Figure 6

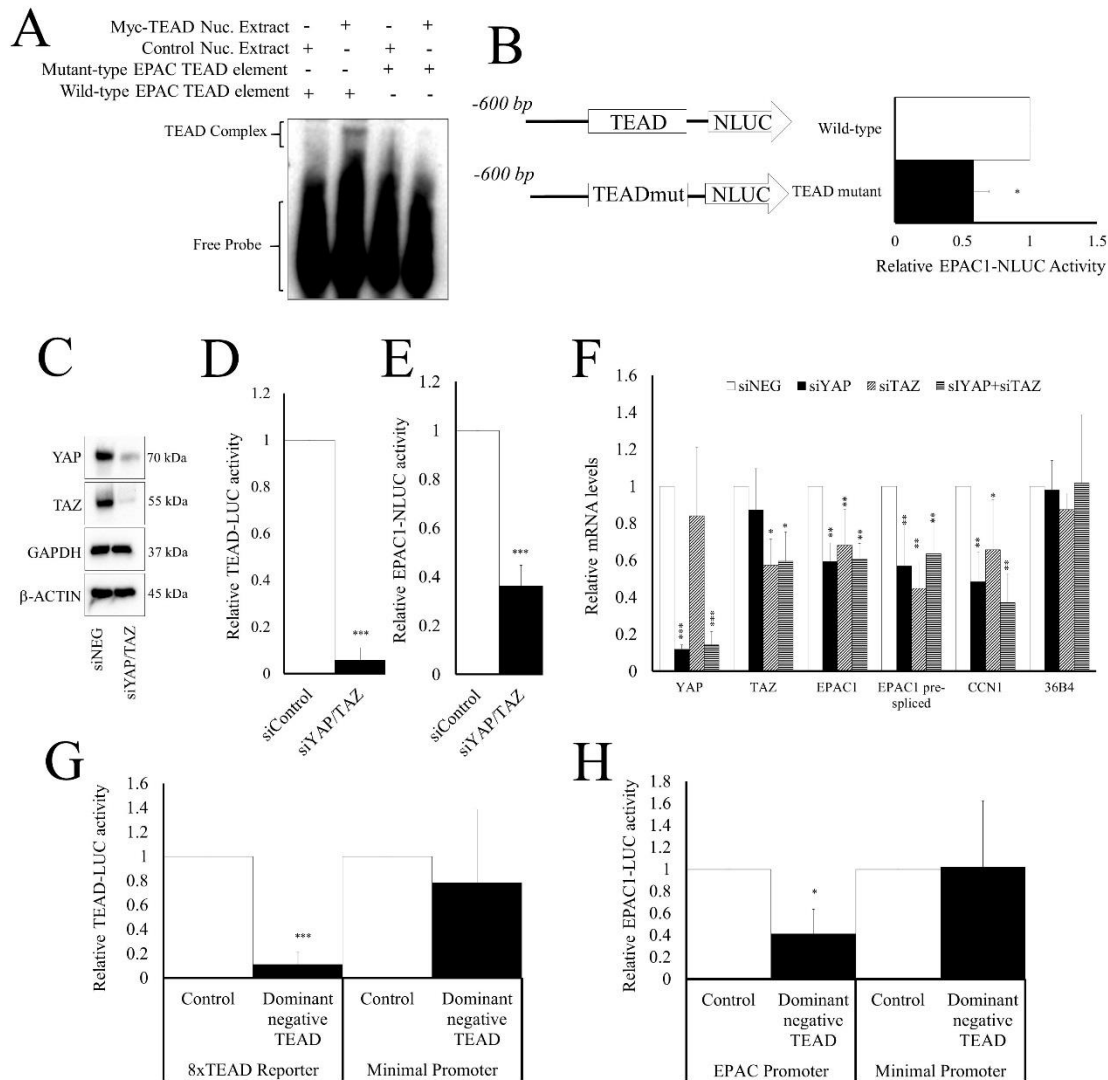


Figure 6: YAP/TAZ-TEAD activity is required for maximal EPAC1 expression

Electromobility shift assay (EMSA) analysis of binding of TEAD protein from nuclear extracts from control or myc-TEAD1 transfected cardiac fibroblasts to DNA probes containing either the wild-type or mutated TEAD element from the EPAC1 proximal promoter (A). Cardiac fibroblasts were transfected with a secreted nanoluciferase reporter (NLUC) gene plasmid under the control of either the wild-type EPAC1 promoter or the EPAC1 promoter containing a mutated TEAD element. Secreted NLUC activity was quantified 24 hours after transient transfection (B). Cardiac fibroblasts were transfected with siRNA targeting YAP and TAZ (siYAP/TAZ) or non-targeting control siRNA (siControl), together with TEAD-LUC or EPAC1-NLUC reporter plasmids. Total cell lysates were analysed for protein levels of YAP, TAZ, GAPDH or β-ACTIN by Western blotting (C) and reporter gene activity quantified (D and E). Total RNA was extracted and analysed for the mRNA levels of YAP, TAZ, EPAC1, pre-spliced EPAC1 mRNA, CCN1 and 36B4 using RT-qPCR (F). Cardiac fibroblasts were transfected with TEAD-LUC (G), EPAC1-NLUC (H) or a minimal promoter reporter (G and H), together with either an empty expression vector lacking a transgene (Control) or a dominant-negative TEAD expression vector, as indicated (F and G). Reporter gene activity was quantified 24 hours after transfection. Data are expressed

as mean \pm SD and analysed by paired Students t-test (B, D, E, G, H) or ANOVA with Student Newman Keul's post-test (F). *, ** and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively.

Figure 7

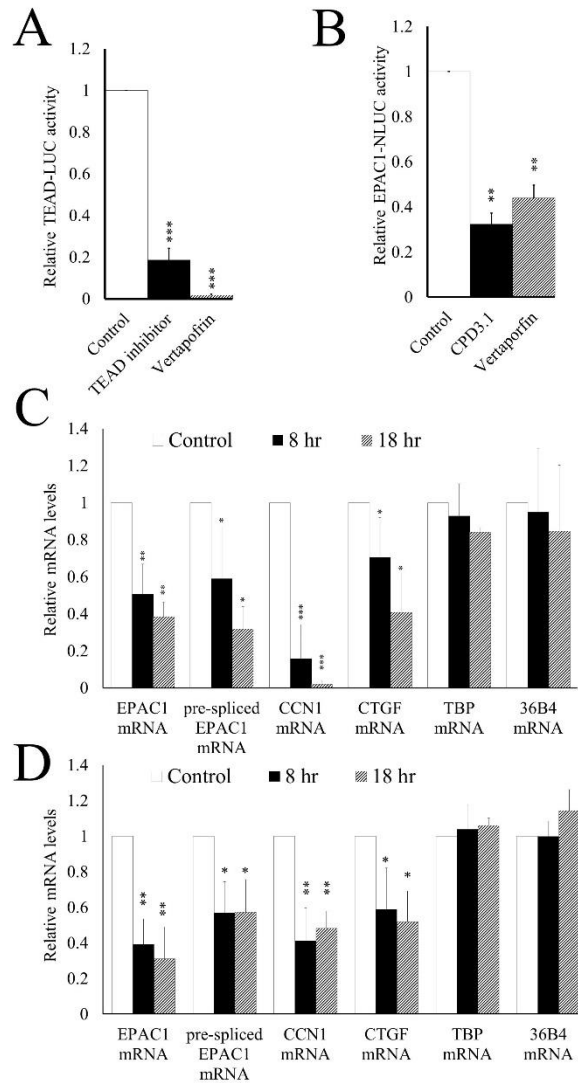


Figure 7: Pharmacological TEAD inhibition reduces EPAC1 expression

Cardiac fibroblasts were transfected with TEAD-LUC reporter (A) or EPAC1-NLUC reporter (B) and stimulated with either 60 μ M CPD 3.1 or 10 μ M verteporfin. Reporter gene activity was quantified 24 hours after transfection. Cardiac fibroblasts were stimulated with 60 μ M CPD3.1 for 8 or 18 hours (C) or 10 μ M Verteporfin (D) and total RNA was extracted and analysed for the mRNA levels of EPAC1, pre-spliced EPAC1, CCN1, CTGF, TBP and 36B4 mRNA by RT-qPCR. Data are expressed as mean \pm SD and analysed by ANOVA with Student Newman Keul's post-test. *, ** and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively.

Figure 8

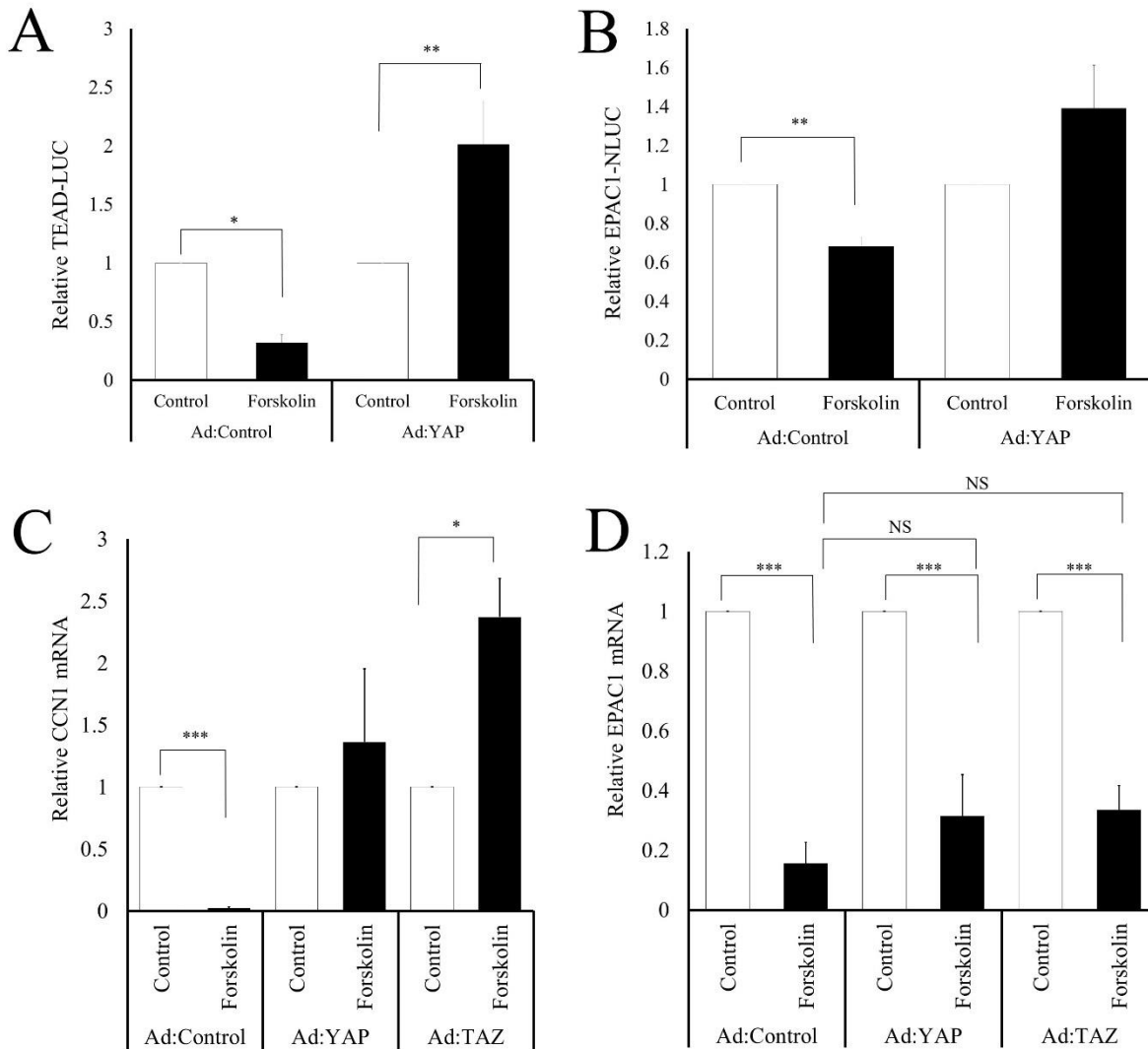


Figure 8: YAP and TAZ are insufficient for EPAC1 mRNA expression

Cardiac fibroblasts were transfected with TEAD-LUC (A) or EPAC-NLUC (B) reporter gene plasmids. The next day, cells were infected with either a control adenovirus (Ad:Control) or adenovirus expressing constitutively-active YAP or TAZ, as indicated (A-D). Cells were then stimulated with 25 μ M forskolin for 8 hours and reporter gene activity quantified (A and B) or total RNA analysed by qRT-PCR for EPAC1 mRNA or CCN1 mRNA levels as indicated. (C and D). Data are expressed as mean \pm SD and analysed by ANOVA with Student Newman Keul's post-test. *, ** and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively.

Figure 9

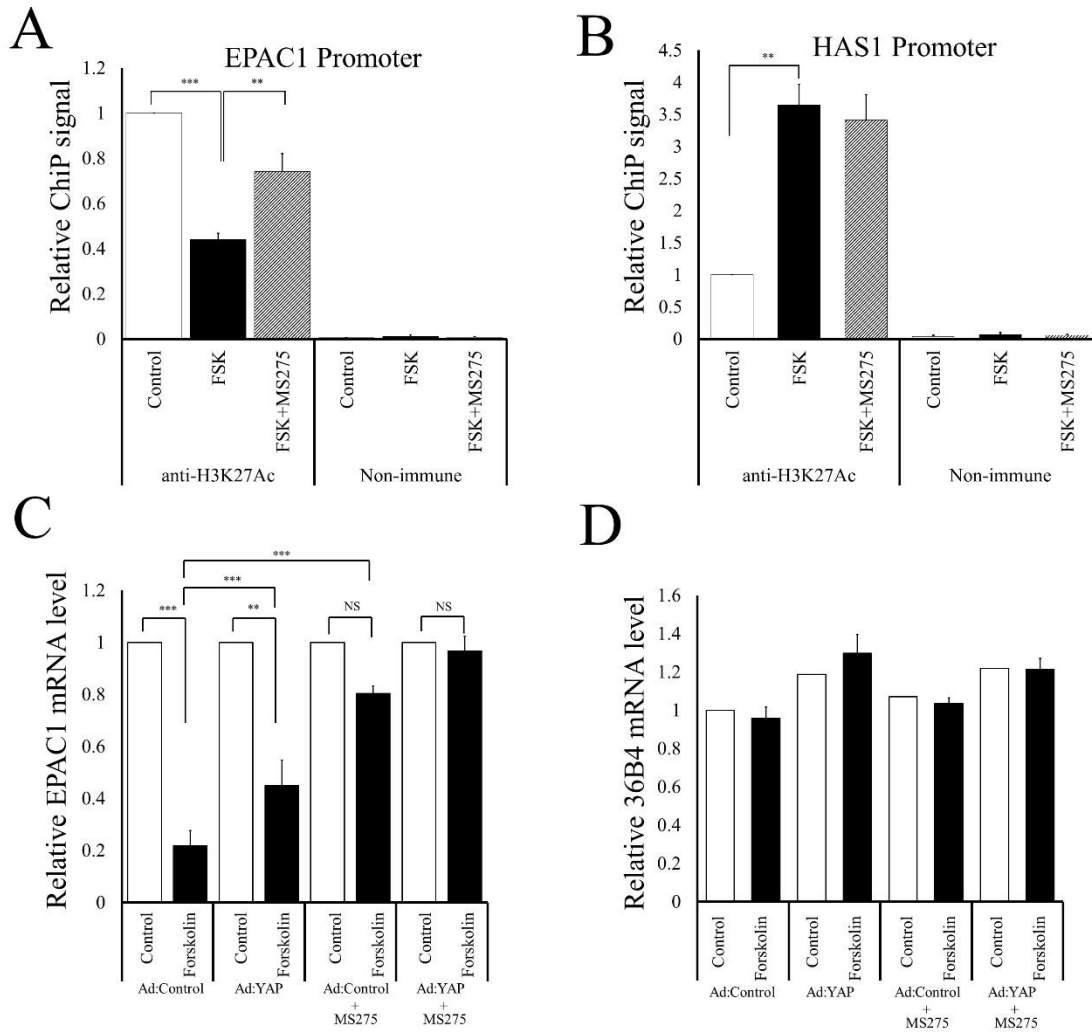


Figure 9: HDAC1/3 mediated deacetylation of histone-3 lysine-27 contributes to cAMP-mediated inhibition of EPAC1 mRNA expression

Cardiac fibroblasts were treated with 5 μ M MS275 for 18 hours followed by stimulation with 25 μ M forskolin for a further 2 hours (A and B). Cells were fixed and chromatin extracted and analysed for H3K27Ac at the proximal EPAC1 (A) or HAS1 (B) promoter regions. Cells were infected with control adenovirus (Ad:Control) or adenovirus expressing constitutively active YAP (Ad:YAP) (C and D). The next day, cells were treated with 5 μ M MS275 for 18 hours followed by stimulation with 25 μ M forskolin for a further 2 hours. Total RNA was extracted and analysed by RT-qPCR for EPAC1 (C) or 36B4 (D) mRNA levels. Data are expressed as mean \pm SD and analysed by ANOVA with Student Newman Keul's post-test. ** and *** indicate, $p < 0.01$ and $p < 0.001$ respectively.

Figure 10

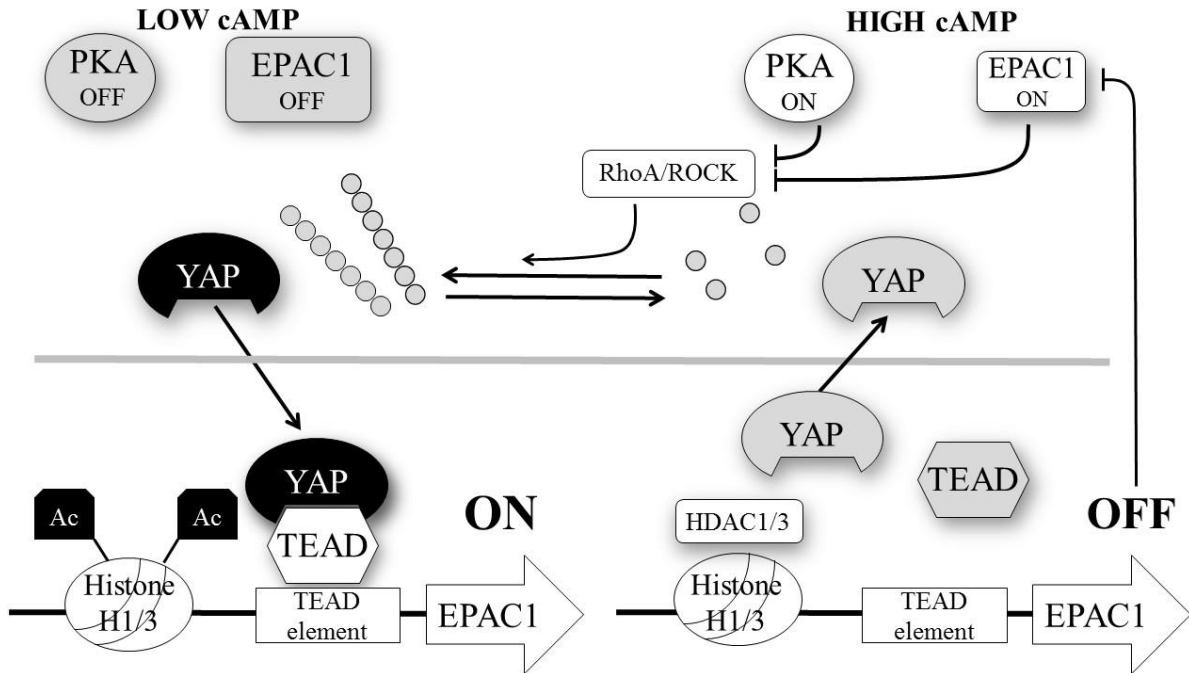


Figure 10: Proposed mechanism underlying cAMP-dependent repression of EPAC1 expression.

In cardiac fibroblast with low levels of cAMP, PKA and EPAC activity are low and RhoA/ROCK activity is high. This results in actin polymerisation and nuclear translocation of YAP/TAZ. YAP/TAZ increase activity of TEAD transcription factors, which act via a conserved TEAD binding element in the proximal EPAC1 promoter to induce EPAC1 gene expression. Elevated cAMP levels activate PKA and EPAC1, which cooperate to inhibit RhoA/ROCK-mediated actin polymerisation, resulting in reduced actin polymerisation and acquisition of a stellate cell morphology. This induces nuclear export of YAP/TAZ, which in turn reduces TEAD activity. Elevated cAMP also induces a HDAC1/3-mediated reduction in histone H3K27 acetylation at the proximal EPAC1 promoter. The reduction in TEAD activity together with the reduction in histone H3K27 acetylation inhibits EPAC1 gene expression.